Molecular Phylogenetics and Evolution 68 (2013) 357–372

Contents lists available at SciVerse ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Testing the use of ITS rDNA and protein-coding genes in the generic and species delimitation of the lichen genus *Usnea* (Parmeliaceae, Ascomycota)

Camille Truong [a,b,*], Pradeep K. Divakar [c], Rebecca Yahr [d], Ana Crespo [c], Philippe Clerc [a,b]

[a] *Conservatoire et Jardin botaniques de la Ville de Genève, CP 60, 1292 Chambésy, Switzerland*
[b] *Laboratoire de systématique végétale et biodiversité, Faculté des sciences de l'Université de Genève, CP 60, 1292 Chambésy, Switzerland*
[c] *Departamento de Biología Vegetal II, Facultad de Farmacia, Universidad Complutense, 28040 Madrid, Spain*
[d] *Royal Botanic Garden Edinburgh, 20A Inverleith Row, Edinburgh EH3 5LR, United Kingdom*

ARTICLE INFO

Article history:
Received 14 June 2012
Revised 1 February 2013
Accepted 2 April 2013
Available online 16 April 2013

Keywords:
Lichen-forming fungi
Ribosomal genes
Protein-coding genes
Sequence alignment
Parallel evolution
Cryptic species

ABSTRACT

In lichen-forming fungi, traditional taxonomical concepts are frequently in conflict with molecular data, and identifying appropriate taxonomic characters to describe phylogenetic clades remains challenging in many groups. The selection of suitable markers for the reconstruction of solid phylogenetic hypotheses is therefore fundamental. The lichen genus *Usnea* is highly diverse, with more than 350 estimated species, distributed in polar, temperate and tropical regions. The phylogeny and classification of *Usnea* have been a matter of debate, given the lack of phenotypic characters to describe phylogenetic clades and the low degree of resolution of phylogenetic trees. In this study, we investigated the phylogenetic relationships of 52 *Usnea* species from across the genus, based on ITS rDNA, nuLSU, and two protein-coding genes *RPB1* and *MCM7*. ITS comprised several highly variable regions, containing substantial genetic signal, but also susceptible to causing bias in the generation of the alignment. We compared several methods of alignment of ITS and found that a simultaneous optimization of alignment and phylogeny (using BAli-phy) improved significantly both the topology and the resolution of the phylogenetic tree. However the resolution was even better when using protein-coding genes, especially *RPB1* although it is less variable. The phylogeny based on the concatenated dataset revealed that the genus *Usnea* is subdivided into four highly-supported clades, corresponding to the traditionally circumscribed subgenera *Eumitria*, *Dolichousnea*, *Neuropogon* and *Usnea*. However, characters that have been used to describe these clades are often homoplasious within the phylogeny and their parallel evolution is suggested. On the other hand, most of the species were reconstructed as monophyletic, indicating that combinations of phenotypic characters are suitable discriminators for delimitating species, but are inadequate to describe generic subdivisions.

1. Introduction

Lichens are mutualistic associations between a fungus and a population of photoautotrophic algae and/or cyanobacteria. They involve one-fifth of all known extant fungal species (Lutzoni et al., 2001). Phenotypic characters that have traditionally been used to characterize the taxonomy of the mycobiont include the morphology of the lichen thallus, the anatomy of reproductive and vegetative structures, or the identification of secondary metabolites (Culberson, 1969; Lumbsch, 1998). Recently, molecular data have had a huge impact on the classification and taxonomy of lichen-forming fungi, with traditional taxonomical concepts frequently in conflict with phylogenetic reconstructions (Crespo et al., 2010b; Lumbsch, 2007; Printzen, 2010). In particular, the parallel evolution of characters, where unrelated groups may share several phenotypic characters that have appeared independently, has been documented among distant lineages of lichen forming-fungi (Baloch et al., 2010; Lutzoni et al., 2004; Schmitt et al., 2009b), as well as at higher levels (Crespo et al., 2007, 2011; Grube and Kantvilas, 2006; Rivas-Plata et al., 2011; Rivas-Plata and Lumbsch, 2011; Tehler and Irestedt, 2007), and identifying appropriate taxonomic characters in accordance with phylogenetic relationships remains challenging in many groups (Crespo and Pérez-Ortega, 2009).

The Parmeliaceae is a highly diverse family of lichenized-fungi within the Lecanorales (Ascomycota), accommodating approximately 2500 species distributed in 85 genera (Crespo et al., 2007). *Usnea* is one of the largest genera within the Parmeliaceae, with more than 350 estimated species. It is characterized by a fruticose, pendulous or erect thallus, attached to the substrate by a short trunk, with cylindrical branches, holding the structures of sexual (apothecia) or vegetative (soralia) reproduction. In section,

---

* Corresponding author at: Conservatoire et Jardin botaniques de la Ville de Genève, CP 60, 1292 Chambésy, Switzerland. Fax: +41 22 4185101.

E-mail address: camille.truong@ville-ge.ch (C. Truong).

1055-7903/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ympev.2013.04.005

branches are composed of a cartilaginous central axis, a medulla formed by loose to compact hyphae, and an outer cortical layer, containing a yellow pigment called usnic acid. Most species grow on bark (corticolous) and can occasionally be found on rocks (saxicolous) or on dead wood (lignicolous), when the environmental conditions are optimal. A few species are primarily saxicolous (Clerc and Herrera-Campos, 1997; Walker, 1985). *Usnea* is a cosmopolitan genus, widely distributed in polar, temperate and tropical regions. The circumscription of species remains extremely complicated due to the exceptional morphological plasticity of the species. Since Motyka's world monograph (1936), taxonomic revisions have been ongoing in Europe (Clerc, 1987, 2011; Halonen et al., 1999), Macaronesia (Clerc, 2006), North America (Clerc, 2008; Halonen et al., 1998; Herrera-Campos et al., 1998, 2001), South America (Rodriguez et al., 2011; Truong et al., 2011; Truong and Clerc, 2012, 2013), East Africa (Swinscow and Krog, 1978, 1979), Eastern Asia (Ohmura, 2001, 2012; Ohmura et al., 2010), India (Awasthi, 1986), Australia (Stevens, 1999, 2004) and polar regions (Seymour et al., 2007; Walker, 1985; Wirtz et al., 2008, 2012).

Usnea forms a strongly supported monophyletic lineage within the family Parmeliaceae (Crespo et al., 2007), but the relationships with sister lineages within the family are not yet resolved. The circumscription of the genus *Usnea* has been a matter of debate for decades. Several entities have been described and their rank as sub-genera or genera have been controversial even before the use of molecular markers: *Neuropogon* (Nees von Esenbeck and Flotow, 1835), *Eumitria* (Stirton, 1882), *Dolichousnea* (Ohmura, 2001, 2002), *Lethariella* and *Protousnea* (Motyka, 1936), the last two being later considered as separate genera since Krog (1976). Articus (2004) proposed to elevate *Dolichousnea*, *Eumitria* and *Neuropogon* at the genus level based on phylogenetic lineages, but other authors agreed to keep these species within the genus *Usnea*, since the backbones of the phylogeny were not fully resolved and the characters used to described these lineages were ambiguous (Ohmura, 2002; Ohmura and Kanda, 2004; Wirtz et al., 2006).

Species boundaries in *Usnea* seem to be relatively well delimited based on the correlation of morphological and molecular characters (Kelly et al., 2011; Ohmura, 2008; Saag et al., 2011), but the presence of several lineages within the same species or the polyphyly of species circumscribed based on morphological characters have also been detected, especially within *Neuropogon* (Lumbsch and Wirtz, 2011; Seymour et al., 2007; Wirtz et al., 2008, 2012). With the introduction of molecular data, the existence of cryptic, morphologically identical species (Bickford et al., 2007) have challenged the traditional view of species delimitation in lichens and have recently received particular consideration in the family Parmeliaceae (Crespo and Lumbsch, 2010; Crespo and Pérez-Ortega, 2009; Del Prado et al., 2010, 2011; Leavitt et al., 2011a). Several new taxa have been recognized based solely on molecular characters (Molina et al., 2011b), or in correlation with overlooked morphological characters (Argüello et al., 2007), secondary metabolites (Molina et al., 2011a), ecology (Molina et al., 2004) or biogeography (Divakar et al., 2010a; Hodkinson and Lendemer, 2011). Similar cases of cryptic speciation have also been demonstrated within other families of lichen-forming fungi (Divakar et al., 2007; Otalora et al., 2010; Parnmen et al., 2012; Spribille et al., 2011). On the other hand, an overestimation of the species diversity based on morphology and chemistry have also been demonstrated (Leavitt et al., 2011b; Velmala et al., 2009).

In phylogenetic studies, the selection of suitable markers for the reconstruction of reliable phylogenetic hypotheses is fundamental. The internal transcribed spacer ITS rDNA is widely used in phylogenetic studies of plants (Poczai and Hyvönen, 2010) and particularly fungi (Schoch et al., 2012). The genus *Usnea* is not an exception (Articus, 2004; Ohmura, 2002, 2008; Ohmura and Kanda, 2004; Saag et al., 2011; Wirtz et al., 2006). With its high mutation rate, ITS provides a signal with a high inter- and intraspecific genetic variability with relatively easy laboratory practices, due to the presence of a high number of copies within the genome and the small size of the target DNA fragment (Schoch et al., 2012). In *Usnea* as in many fungi, amplification by PCR of ribosomal DNA has a much greater success than when targeting protein-coding genes. However, the use of ITS includes several drawbacks, such as the potentiality for paralogues, high levels of homoplasy, and the generation of sequence alignments with low confidence (Alvarez and Wendel, 2003; Poczai and Hyvönen, 2010). Other markers include single copy protein-coding genes, such as the RNA polymerases (*RPB1* and *RPB2)* that have been successfully used to obtain highly supported phylogenies (Crespo et al., 2007, 2010b; Hofstetter et al., 2007; Lumbsch et al., 2007), but also to discriminate among closely related species of lichenized (Nelsen et al., 2011; Savic and Tibell, 2009; Wirtz et al., 2008) and non-lichenized (Matheny et al., 2002; Schoch et al., 2012) fungi. Another example is *MCM7*, a DNA replication licensing factor that has been recently developed in phylogenetic studies of lichenized fungi (Divakar et al., 2012; Leavitt et al., 2011a,b; Raja et al., 2011; Schmitt et al., 2009a). Spribille et al. (2011) reported that the phylogenetic signal provided by *MCM7* was comparable to that of ITS for discriminating among species.

Sequence alignment is one critical step in molecular phylogenetic analysis (Blackburne and Whelan, 2013; Blair and Murphy, 2011; Ogden and Rosenberg, 2006), although the priority is often placed in tree building (Morrison, 2009). A conservative approach is usually the exclusion of ambiguously aligned regions from the alignment (Lutzoni, 1997; Lutzoni et al., 2000). However it is recognized that these ambiguous sites carry a substantial phylogenetic signal (Redelings and Suchard, 2009) that is especially informative at the species level (Gaya et al., 2011; Lutzoni et al., 2000). Several analytical methods have been developed to capture this signal without violating positional homology: ambiguous regions can be processed through a coding procedure and the resulting mixed matrix of DNA (aligned regions) and codes (ambiguous regions) is analyzed with maximum parsimony (Lutzoni et al., 2000) or maximum likelihood criteria (Lücking et al., 2011). Another approach simultaneously optimizes both the alignment and the phylogeny using a Bayesian inference (Redelings and Suchard, 2005). Unlike other methods that base phylogeny estimates on a single fixed alignment, alignment uncertainty is taken into account by considering all possible alignments. Several studies recently demonstrated that this approach was the most efficient to resolve phylogenetic relationships among species of lichen-forming fungi based on ITS sequences (Gaya et al., 2011; Śliwa et al., 2012).

In this study we used multi-locus data to reconstruct the phylogeny of the genus *Usnea* and evaluate morphologically circumscribed species in the light of molecular data. We then used this multi-locus best-estimate of the phylogeny as a measure of the genetic information of the ITS region from several methods of alignment; (1) a strict alignment with ambiguous sites removed following GUIDANCE (Penn et al., 2010); (2) a relaxed alignment obtained with Gblocks (Castresana, 2000); (3) a mixed alignment of DNA and ambiguous sites coded with PICS-Ord (Lücking et al., 2011); and (4) a joint estimation of alignment and phylogeny inferred with BAli-phy (Redelings and Suchard, 2005).

2. Material and methods

2.1. Taxon sampling

Material from 110 *Usnea* specimens representing 52 species from across the genus, originating from polar, temperate and